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# Influence of chronic alcoholism and oestrogen deficiency on the variation of stoichiometry of hydroxyapatite within alveolar bone crest of rats☆

*Adriana M.P.S. Marchini* [a,*], *Camila P. Deco* [a], *Karina B. Lodi* [a], *Leonardo Marchini* [a,b,c], *Ana M.E. Santo* [d,e], *Rosilene F. Rocha* [a]

[a] *Department of Biosciences and Diagnostics, São José dos Campos School of Dentistry, São Paulo State University – UNESP, São José dos Campos, São Paulo, Brazil*
[b] *School of Dentistry, University of Vale do Paraíba – UNIVAP, São José dos Campos, São Paulo, Brazil*
[c] *Department of Dentistry, University of Taubaté – UNITAU, Taubaté, São Paulo, Brazil*
[d] *Institute of Research and Development – IP&D, Laboratory of Biomedical Vibrational Spectroscopy, University of Vale do Paraíba, São José dos Campos, São Paulo, Brazil*
[e] *Department of Mathematical and Earth Sciences, Federal University of São Paulo – UNIFESP, Diadema, São Paulo, Brazil*

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ABSTRACT

*Objective:* Previous findings suggest that chronic alcoholism and oestrogenic deficiency may affect bones in general (including alveolar bone) and increase individuals' susceptibility to the development of periodontal disease. The aim of this study was to assess possible alterations in the chemical composition of alveolar bone in rats subjected to chronic alcoholism, oestrogen deficiency or both.

*Design:* Fifty-four rats were initially divided into two groups: ovariectomized (Ovx), and Sham operated (Sham). A month after surgery, the groups were sub-divided and received the following dietary intervention for eight weeks: 20% alcohol, isocaloric diet and ad libitum diet. Samples of the mandible, in the alveolar bone crest region, were analyzed to verify possible changes in the stoichiometric composition of bone hydroxyapatite, by measuring the relationship between the concentration of calcium and phosphorus (Ca/P ratios), using micro X-ray fluorescence spectrometry.

*Results:* The ad libitum groups presented the highest average values of Ca/P ratios, while the groups with dietary restrictions presented the smallest average values. The Ovx ad libitum group presented the highest values of Ca/P ratios ($2.03 \pm 0.04$). However, these values were not considered statistically different ($p > 0.05$) from the Sham ad libitum group ($2.01 \pm 0.01$). The Ovx alcohol group presented lower values for Ca/P ratios ($1.92 \pm 0.06$), being the only group statistically different ($p < 0.001$) from the Sham ad libitum group. Potential confounding variables are discussed.

*Conclusion:* Ovariectomy associated with alcohol consumption at 20% significantly changed the stoichiometry composition of hydroxyapatite in the alveolar bone crest, leading to a reduction in Ca/P ratios.



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* *Corresponding author at*: Av. Adhemar de Barros, 1136/153, São José dos Campos, SP 12245-010, Brazil. Fax: +55 12 39221555.
E-mail address: adrianampsmarchini@gmail.com (Adriana M.P.S. Marchini).
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## 1. Introduction

Bones are composed of mineralized tissue constituting mainly of calcium (Ca) and phosphorous (P). These elements are organized forming crystals of hydroxyapatite.[1] Some conditions or pathologies affecting this tissue may alter the quantitative distribution of these elements, and consequently the stoichiometric composition of hydroxyapatite.[2–4]

Osteoporosis is a metabolic bone disorder, the most frequent etiologic factor of which is oestrogen deficiency, which occurs mainly in women after menopause.[5] This condition causes changes in the pattern of bone remodelling, with a predominance of the resorption process, which can alter the homeostasis of Ca and P and decrease bone mineral density.[4,6,7] Despite the importance of oestrogen deficiency in the aetiology of osteoporosis, it is a multi-factorial disease, involving several other risk factors, including excessive consumption of alcohol.[8]

The effects of abusive alcohol consumption on bone quality seem to be more dramatic in young individuals.[9] However, a decrease in bone mineral density when alcohol is consumed in large quantities, has also been reported in women after menopause.[10]

Periodontal disease is an infectious immune inflammatory alteration that affects the structures which support teeth. The primary etiological factor of which is bacterial biofilm.[11] However, its progression may be influenced by a wide range of variables which include systemic diseases (e.g. diabetes and osteoporosis), genetic disorders, habits (e.g. smoking and/or alcoholism), age, gender, stress, nutritional problems, including other factors, which may influence the way the host responds to an aggressive agent.[12–18]

Literature reviews have suggested that osteoporosis associated with both oestrogen deficiency and excessive alcohol consumption can be considered potential risk factors for the development of periodontal disease, which, if not controlled, could lead to tooth loss. However, the information available in the literature is insufficient for a definitive consensus, which highlights the need for further research by undertaking a greater number of well controlled and longitudinal studies.[15,16,19,20] It is possible that systemic bone loss associated with osteoporosis/osteopenia can also affect alveolar bone and its porosity which would lead to a greater susceptibility of bone resorption in the region.[15]

Despite the importance of Ca and P as major constituents of bone mineral phase and the possible implications of oestrogen deficiency and excessive alcohol consumption on the development of periodontal disease, to the best of our knowledge there are no studies that have evaluated concentrations of these chemical elements under these conditions, specifically in the region of alveolar bone crest, a structure whose integrity is important for the maintenance of periodontal health.

Considering the absence of such studies, this paper aims to evaluate the effect of oestrogen deficiency and excessive alcohol consumption on alveolar bone crest. Considering previous findings regarding oestrogen deficiency and alcohol consumption,[21–23] the hypothesis of this study is that oestrogen deficiency associated with alcohol consumption can adversely influence the quality of alveolar bone and alter its mineral composition.

## 2. Materials and methods

### 2.1. Treatment of animals

The present study was approved by the ethics committee of São José dos Campos School of Dentistry, State University of São Paulo – UNESP (Protocol No. 021/2008-PA/CEP).

Fifty-four rats (*Rattus norvegicus*, of the albinus, Wistar variety), aged four-months, were initially divided into two groups: ovariectomized (rats subjected to oestrogen deficiency by removing the ovaries), and Sham operated (simulated ovariectomy, ovaries exposed but not removed). A month after surgery, the two groups were sub-divided, and received the following dietary intervention for eight weeks: (a) alcoholic diet: solid diet and a 20% alcohol solution ad libitum, (b) isocaloric diet: solid and liquid diets with the same amount of calories consumed by the alcohol group and (c) ad libitum diet: solid diet and water ad libitum. The animals were randomized by weight in their respective groups.

The 20% alcohol solution was obtained by an absolute alcohol dilution in water. The concentration of the isocaloric solution contained, in millilitres, the same amount of calories as the 20% alcohol solution. It was prepared by dissolving 266 g sucrose in 1 l of water. Calculations were made taking into account the alcohol concentrations (20%), the density of absolute alcohol (0.787 g/ml) and the caloric values of sucrose (4.1 kcal/g) and alcohol (7.1 kcal/g). The solid diet was a commercial food (Labina – Purina®, Paulínia, Brazil).

The amount of calories (solid diet and alcohol solution) ingested by animals in the alcohol groups was measured daily. The following day, a diet with the same amount of calories (solid diet and isocaloric solution) was offered to isocaloric groups. Doing so, the treatment of animals with the isocaloric diet began and finished a day after the groups with the alcoholic diet.

To prevent dehydration, animals from the isocaloric groups also received water ad libitum. These animals received two bottles, one containing the sucrose solution and the other, solely water. However, in the statistical analysis of fluid consumption, for the isocaloric groups, only the amount of ingested sucrose solution was considered. This was done, as our intention was to compare the amount of calories ingested by the different experimental groups.

In summary, during the dietary treatment, the rats were divided into six experimental groups (each one presenting $n = 9$): Sham operated and ad libitum diet (Sham/ad libitum); ovariectomized and ad libitum diet (Ovx/ad libitum); Sham operated and alcoholic diet (Sham/alc); ovariectomized and alcoholic diet (Ovx/alc); Sham operated and isocaloric diet (Sham/iso); and ovariectomized and isocaloric diet (Ovx/iso). The Sham/iso group was pair-fed to Sham/alc group, while the Ovx/iso group was pair-fed to the Ovx/alc group.

Eight weeks after commencing the dietetic treatment, the rats were sacrificed and their mandibles removed. The ovariectomy success was confirmed, after sacrifice, by the visualization of ovary absence and uterus atrophy.

The rats were weighed at the beginning and at the end of the experiment. Weight changes were observed in percentage according to the formula below:

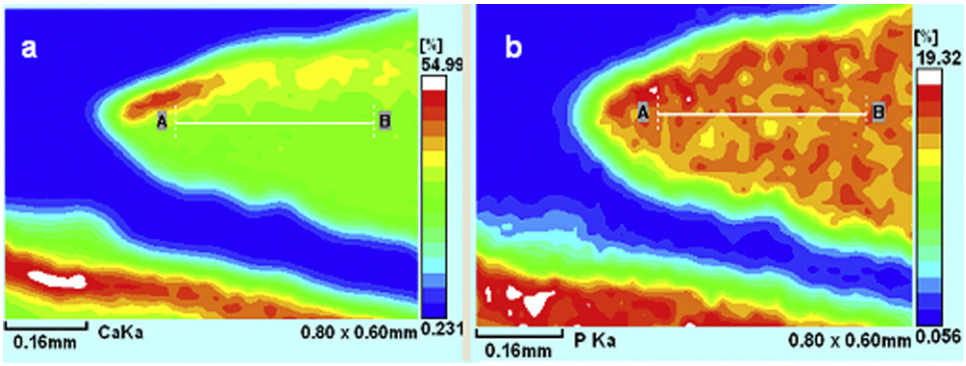


**Fig. 1 – Line AB of 0.3 mm, drawn at the centre of the bone crest, approximately 0.1 mm below the tip of the crest. Sample Sham/ad libitum group. (a) Mapping of calcium and (b) mapping of phosphorus.**

$$\frac{(\text{final weight} - \text{initial weight}) \times 100}{\text{initial weight}}$$

The average value of solid and liquid diet consumed per rat/per day was recorded.

### 2.2. *X-ray fluorescence spectrometry*

The amount of Ca and P and the relative ratio of Ca/P, present in the alveolar bone crest, were measured using an energy-dispersive micro X-ray fluorescence spectrometer (μEDX 1300 – 50 μm – Shimadzu®, Kyoto, Japan).

After sacrifice, the mandibles were placed in a solution of 10% buffered formalin for 24 h, washed with water, then dried and frozen at −20 °C. Fixation of biological samples in formaldehyde based solutions prior to the analyses of concentrations of Ca and P in bone had already been undertaken by other authors.[24–26] To reduce possible interference to the fixation procedure in the interpretation of the results, all samples were fixed for the same period of time. The fixation in formalin was done to prevent the putrefaction of the samples during the spectrometric analysis.

The region of the alveolar bone crest, right side of the mandible, between the 1st and 2nd molar, were flattened using sandpaper no. 1200 coupled to an automatic polishing machine. This was necessary as irregularities on the surface of the sample could influence the interaction of electrons and the propagation of X-rays.

The samples were mapped on a rectangular area, including the alveolar bone crest, which led to a window of 0.80 mm × 0.60 mm (40 × 30 points with increments of 20 μm). The voltage was set at 15 kV with automatic adjustment of the current. The time required for the mapping of each sample was approximately 260 min. The calibration of the equipment used for reference, was a commercial reagent of synthetic hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$ – 99.999% grade – Sigma–Aldrich®, St. Louis, USA). The Ca/P ratio calculated (theoretically), in weight percentage, used to compare the results was 2.16, calculated from the stoichiometry. The calculations were obtained considering 10 mol of Ca with molar mass of 40.08 g/mol and 6 mol of P with molar mass 30.97 g/mol.

After obtaining the image of the map, a line of 0.3 mm was drawn at the centre of the bone crest, approximately 0.1 mm below the tip of the crest, in which the average concentrations of Ca and P were obtained. These averages were used to perform the calculation of the Ca/P ratios (Fig. 1).

### 2.3. *Statistical analysis*

Data concerning the weight and diet of the rats showed non-normal distribution and were performed using non-parametric tests (Kruskal–Wallis and Mann–Whitney). No statistical adjustment was applied to the samples.

Data obtained by spectrophotometry were normally distributed and were analyzed by analysis of variance (ANOVA) and Tukey test.

All comparisons were made with a significance level of 5%.

## 3. Results

### 3.1. *Diet and weight*

The values of weight changes by percentage, solid diet and liquid diet are compared and described in Tables 1 and 2 (Kruskal–Wallis and Mann–Whitney).

In all groups there was an average weight gain in the rats during the experiment. The group that gained more weight, by percentage, was Ovx/ad libitum (average gain of 30.9 ± 12.6%). This group was considered statistically different from all other groups (Tables 1 and 2). Table 3 shows the weight of the animals by absolute value, comparing the initial values (at the time of ovariectomy or Sham surgery) to those of the end of the experiment (sacrifice).

The group that consumed the more solid diet was Ovx/ad libitum (18.46 ± 1.46 g), which was statistically different from all other groups except for the Sham/ad libitum. Although there was no statistical difference between the Sham/ad libitum and Ovx/ad libitum, one must consider that the *p*-value is very close to the acceptable limit, which indicates a trend towards difference ($p = 0.058$). It was noted also that the isocaloric groups consumed all the solid diet offered to them. This meant that solid food consumption

**Table 1 – Analysis comparing weight changes in percentage, solid diet and liquid diet (Kruskal–Wallis test).**

| Groups | Sham/ad libitum | Ovx/ad libitum | Sham/alc | Ovx/alc | Sham/iso | Ovx/iso |
|---|---|---|---|---|---|---|
| *Weight (% gain)* | | | | | | |
| Average | 19.9% | 30.9% | 15.9% | 13.9% | 11.1% | 17.3% |
| Median | 19.8% | 27.8% | 18.8% | 15.0% | 11.5% | 14.8% |
| Standard deviation | 6.2% | 12.6% | 5.1% | 9.2% | 5.0% | 11.3% |
| Confidence interval | 4.3% | 8.7% | 3.4% | 6.0% | 3.3% | 7.4% |
| Sample size (*n*)[b] | 8 | 8 | 9 | 9 | 9 | 9 |
| *p* Value | 0.003[a] | | | | | |
| *Solid diet (g/day/rat)* | | | | | | |
| Average | 16.84 | 18.46 | 12.91 | 12.81 | 12.91 | 12.81 |
| Median | 16.19 | 18.48 | 12.62 | 12.60 | 12.62 | 12.60 |
| Standard deviation | 2.08 | 1.46 | 1.58 | 1.44 | 1.58 | 1.44 |
| Confidence interval | 1.44 | 1.01 | 1.09 | 1.00 | 1.09 | 1.00 |
| Sample size (*n*)[c] | 8 | 8 | 8 | 8 | 8 | 8 |
| *p* Value | <0.001[a] | | | | | |
| *Liquid diet (ml/day/rat)* | | | | | | |
| Average | 32.02 | 24.98 | 17.13 | 16.24 | 10.52 | 11.37 |
| Median | 31.69 | 24.58 | 16.93 | 15.90 | 10.38 | 11.05 |
| Standard deviation | 3.48 | 3.16 | 1.89 | 1.41 | 1.30 | 1.38 |
| Confidence interval | 2.41 | 2.19 | 1.31 | 0.98 | 0.90 | 0.95 |
| Sample size (*n*)[c] | 8 | 8 | 8 | 8 | 8 | 8 |
| *p* Value | <0.001[a] | | | | | |

[a] Value considered statistically significant.
[b] Sample size (*n*) – considering the number of animals in each group.
[c] Sample size (*n*) – considering the average solid or liquid diets ingested during the 8 weeks of treatment.

**Table 2 – Statistical *p* values comparing weight changes in percentage, solid diet and liquid diet (Mann–Whitney test).**

| Groups | Ovx/ad libitum | Sham/alc | Ovx/alc | Sham/iso | Ovx/iso |
|---|---|---|---|---|---|
| *Weight (% gain)* | | | | | |
| Sham/ad libitum | 0.036[a] | NS[b] | NS[b] | 0.009[a] | NS[b] |
| Ovx/ad libitum | | 0.004[a] | 0.009[a] | 0.001[a] | 0.027[a] |
| Sham/alc | | | NS[b] | 0.070[c] | NS[b] |
| Ovx/alc | | | | NS[b] | NS[b] |
| Sham/iso | | | | | NS[b] |
| *Solid diet* | | | | | |
| Sham/ad libitum | 0.058[c] | 0.005[a] | 0.002[a] | 0.005[a] | 0.002[a] |
| Ovx/ad libitum | | 0.001[a] | 0.001[a] | 0.001[a] | 0.001[a] |
| Sham/alc | | | NS[b] | NS[b] | NS[b] |
| Ovx/alc | | | | NS[b] | NS[b] |
| Sham/iso | | | | | NS[b] |
| *Liquid diet* | | | | | |
| Sham/ad libitum | 0.002[a] | 0.001[a] | 0.001[a] | 0.001[a] | 0.001[a] |
| Ovx/ad libitum | | 0.001[a] | 0.001[a] | 0.001[a] | 0.001[a] |
| Sham/alc | | | NS[b] | 0.001[a] | 0.001[a] |
| Ovx/alc | | | | 0.001[a] | 0.001[a] |
| Sham/iso | | | | | NS[b] |

[a] Statistically significant difference.
[b] Not significant *p* values.
[c] *p* Values which are not significant, but are near the limit of acceptance (trend towards difference).

between Ovx/iso and Ovx/alc and Sham/iso and Sham/alc was equivalent ($12.81 \pm 1.44$ g and $12.91 \pm 1.58$ g, respectively) (Tables 1 and 2).

The following liquids were evaluated: alcohol solution (20%), sucrose solution and water, in relation to alcohol, isocaloric and ad libitum diet groups, respectively. Although the isocaloric groups were offered a sucrose solution equivalent to the alcohol solution (consumed by alcohol groups on the previous day), the rats did not ingest all the solution available to them. The Ovx/alc group ingested an average of $16.24 \pm 1.41$ ml of alcohol solution and the Ovx/iso group ingested $11.37 \pm 1.38$ ml of sucrose solution. The Sham/alc group ingested $17.13 \pm 1.89$ ml of alcohol solution and Sham/iso group ingested $10.52 \pm 1.30$ ml of sucrose solution (Table 1).

Two animals died prior to the end of treatment (one in the Sham/ad libitum group and the other in the Ovx/ad libitum group). The cause of the deaths was unknown.

**Table 3 – Weight of animals by absolute value, comparing their initial weights and that at sacrifice.**

| Weight (g) | Average | Median | Standard deviation | Confidence interval | Number of animals |
|---|---|---|---|---|---|
| *Ovx/alc* | | | | | |
| Initial | 278.9 | 270 | 16.9 | 11.1 | 9 |
| Sacrifice | 317.8 | 325 | 32.4 | 21.2 | 9 |
| *Ovx/iso* | | | | | |
| Initial | 270.6 | 270 | 24.3 | 15.9 | 9 |
| Sacrifice | 316.1 | 310 | 28.0 | 18.3 | 9 |
| *Ovx/ad libitum* | | | | | |
| Initial | 273.1 | 270 | 16.2 | 11.3 | 8 |
| Sacrifice | 356.3 | 355 | 25.7 | 17.8 | 8 |
| *Sham/alc* | | | | | |
| Initial | 262.2 | 260 | 21.7 | 14.2 | 9 |
| Sacrifice | 303.3 | 310 | 19.0 | 12.4 | 9 |
| *Sham/iso* | | | | | |
| Initial | 266.1 | 270 | 21.3 | 13.9 | 9 |
| Sacrifice | 295.0 | 290 | 17.7 | 11.5 | 9 |
| *Sham/ad libitum* | | | | | |
| Initial | 248.1 | 243 | 24.0 | 16.7 | 8 |
| Sacrifice | 297.5 | 290 | 31.3 | 21.7 | 8 |

### 3.2. *Analysis of alcohol consumption*

The average amount of 20% alcohol solution consumed per day per rat was 16.69 ml. With this data it was able to calculate the amount of alcohol consumed in other units of measurement (Table 4).

The amount of absolute alcohol consumed in grams per kilogram of animal weight per day was evaluated. The results showed that on average, the rats consumed 8.76 g of absolute alcohol per kg/day (Table 4).

The percentage of calories from the alcohol diet was also calculated. The results showed that on average 37.83% of dietary calories came from alcohol consumption (Table 4).

### 3.3. *Spectrometric analysis (concentrations of Ca, P and Ca/P ratios)*

During the experiment, some samples were discarded (due to failure of standardization during sample preparation), which altered the final number of samples analyzed by the spectrometer per experimental group (Table 5).

The analysis of the concentrations of Ca, P and Ca/P ratios are shown in Tables 5 and 6 (ANOVA and Tukey test). The highest values in the Ca/P ratios were obtained from the analysis of the Ovx/ad libitum group (mean $2.03 \pm 0.04$), which was considered statistically different from other groups, except the Sham/ad libitum and Sham/alc. The lowest values were obtained in the Ovx/alc group (mean $1.92 \pm 0.06$), which was considered statistically different from other groups, except the Sham/iso. It should be also noted that the Ovx/alc group was the only one to show a statistically significant difference when compared with the Sham/ad libitum group ($p < 0.001$).

## 4. Discussion

Analysis of Ca/P ratios, as compared to the concentrations of Ca and P separately, show lower values of standard deviation and coefficient of variation, which may be more reliable for the diagnosis of bone disorders.[2] In our study, the average values for the Ca/P ratios ranged from $1.92 \pm 0.06$ to $2.03 \pm 0.04$,

**Table 4 – Analysis of alcohol consumption.**

| Alcohol consumption | Sham/alc | Ovx/alc | Average of alcohol groups |
|---|---|---|---|
| Average intake of alcohol solution 20% (ml/day/rat) | 17.13 ml | 16.24 ml | 16.69 ml |
| Average intake of absolute alcohol (ml/day/rat) | 3.43 ml | 3.25 ml | 3.34 ml |
| Average intake of absolute alcohol (g/day/rat)[a] | 2.70 g | 2.56 g | 2.63 g |
| Average intake of absolute alcohol (g/kg of animal weight/day)[b] | 8.99 g | 8.52 g | 8.76 g |
| Feed intake (g/day/rat) | 12.91 g | 12.81 g | 12.86 g |
| Feed intake (kcal/day/rat)[c] | 30.80 kcal | 30.56 kcal | 30.68 kcal |
| Alcohol consumption (kcal/day/rat)[d] | 19.17 kcal | 18.18 kcal | 18.67 kcal |
| % Calorie diet from alcohol (average per day/rat) | 38.36% | 37.30% | 37.83% |

[a] Considering that 1 ml of alcohol is equivalent to 0.787 g.
[b] Considering an animal with an average weight of 300 g.
[c] Considering that 1 g of commercial food (Labina – Purina®, Paulínia, Brazil) has 2.386 kcal.
[d] Considering that 1 g of alcohol is equivalent to 7.1 kcal.

**Table 5 – Analysis for chemical elements contents of Ca and P (in weight percentage), and the Ca/P ratios.**

| Groups | Sample size (*n*) | Average | Standard deviation | Median | CV (%)[a] |
|---|---|---|---|---|---|
| Sham/ad libitum Ca | 7 | 30.39 | 0.76 | 30.47 | 2.50 |
| Ovx/ad libitum Ca | 7 | 31.21 | 0.74 | 31.34 | 2.37 |
| Sham/alc Ca | 7 | 29.92 | 0.86 | 29.63 | 2.87 |
| Ovx/alc Ca | 8 | 27.71 | 1.13 | 27.63 | 4.08 |
| Sham/iso Ca | 7 | 27.76 | 0.48 | 27.69 | 1.73 |
| Ovx/iso Ca | 6 | 28.03 | 0.75 | 28.03 | 2.68 |
| Sham/ad libitum P | 7 | 15.15 | 0.39 | 15.11 | 2.57 |
| Ovx/ad libitum P | 7 | 15.37 | 0.28 | 15.41 | 1.82 |
| Sham/alc P | 7 | 14.97 | 0.41 | 14.86 | 2.74 |
| Ovx/alc P | 8 | 14.44 | 0.39 | 14.35 | 2.70 |
| Sham/iso P | 7 | 14.12 | 0.25 | 14.12 | 1.77 |
| Ovx/iso P | 6 | 14.20 | 0.22 | 14.12 | 1.55 |
| Sham/ad libitum Ca/P | 7 | 2.01 | 0.01 | 2.01 | 0.50 |
| Ovx/ad libitum Ca/P | 7 | 2.03 | 0.04 | 2.02 | 1.97 |
| Sham/alc Ca/P | 7 | 2.00 | 0.01 | 2.00 | 0.50 |
| Ovx/alc Ca/P | 8 | 1.92 | 0.06 | 1.90 | 3.13 |
| Sham/iso Ca/P | 7 | 1.97 | 0.02 | 1.97 | 1.02 |
| Ovx/iso Ca/P | 6 | 1.97 | 0.03 | 1.98 | 1.52 |

[a] Coefficient of variation by percentage.

**Table 6 – Statistical $p$ values for the concentrations of Ca, P and Ca/P ratios (ANOVA and Tukey test).**

| Groups | Ovx/ad libitum | Sham/alc | Ovx/alc | Sham/iso | Ovx/iso |
|---|---|---|---|---|---|
| Ca | | | | | |
| Sham/ad libitum Ca | NS[b] | NS[b] | $p < 0.001$[a] | $p < 0.001$[a] | $p < 0.001$[a] |
| Ovx/ad libitum Ca | | $p < 0.05$[a] | $p < 0.001$[a] | $p < 0.001$[a] | $p < 0.001$[a] |
| Sham/alc Ca | | | $p < 0.001$[a] | $p < 0.001$[a] | $p < 0.001$[a] |
| Ovx/alc Ca | | | | NS[b] | NS[b] |
| Sham/iso Ca | | | | | NS[b] |
| P | | | | | |
| Sham/ad libitum P | NS[b] | NS[b] | $p < 0.001$[a] | $p < 0.001$[a] | $p < 0.001$[a] |
| Ovx/ad libitum P | | NS[b] | $p < 0.001$[a] | $p < 0.001$[a] | $p < 0.001$[a] |
| Sham/alc P | | | $p < 0.05$[a] | $p < 0.001$[a] | $p < 0.001$[a] |
| Ovx/alc P | | | | NS[b] | NS[b] |
| Sham/iso P | | | | | NS[b] |
| Ca/P | | | | | |
| Sham/ad libitum Ca/P | NS[b] | NS[b] | $p < 0.001$[a] | NS[b] | NS[b] |
| Ovx/ad libitum Ca/P | | NS[b] | $p < 0.001$[a] | $p < 0.05$[a] | $p < 0.05$[a] |
| Sham/alc Ca/P | | | $p < 0.001$[a] | NS[b] | NS[b] |
| Ovx/alc Ca/P | | | | NS[b] | $p < 0.05$[a] |
| Sham/iso Ca/P | | | | | NS[b] |

[a] Statistically significant differences ($p < 0.05$ and $p < 0.001$).
[b] Not significant $p$ values ($p > 0.05$).

smaller than the molar ratio of synthetic hydroxyapatite, which is 2.16. These results were expected, as the bone mineral phase is formed by nonstoichiometric carbonated apatite ionic crystals.[7]

Alterations on bone quality when ovariectomy was associated with alcohol consumption were previously observed by other authors.[21–23,27] In ovariectomized rats who received 20% alcohol solution (similar to that in the present experiment) an exacerbation of bone loss in the alveolar bone crest,[21] decreased Ca/P ratios in the femur (associated with serum hyperphosphataemia)[22] and negative effects on osseointegration[23] was noted. Due to these findings, it was hypothesized above that oestrogen deficiency associated with alcohol consumption can adversely influence the quality of alveolar bone, and change its mineral composition. This hypothesis was confirmed by the presented results, as the Ovx/alc group was the only group that presented statistically different results concerning Ca/P ratio when compared to the control (Sham–ad libitum diet).

Little is known about the influence of mineralization in periodontal disease or tooth loss. In a study[21] with the same experimental design to that of the current study (with respect to animal treatment), it was observed, by histological and histomorphometrical analyses (slides stained with haematoxylin–eosin), an exacerbation of alveolar bone loss and inflammatory process, in periodontal tissues, in ovariectomized rats who received 20% alcohol (group Ovx/alc).[21] In our experiment, it was verified that, under the same conditions, there was a decrease in the values of Ca/P ratios in alveolar bone. Thus, it seems reasonable to assume that there is the

possibility of a reduction in mineralization linked to an increase in alveolar bone loss and the severity of periodontal disease, which could as a consequence compromise tooth retention. However, this is only a hypothesis.

In another experiment, Yan et al.,[26] also noted the influence of mineralization in the region of the periodontium (more specifically in artificially created furcation defects). The authors checked with histological/histomorphometrical analysis, and by energy dispersive X-ray, that higher values in Ca/P could be related to increased rates of periodontal regeneration.

Molecular and cellular mechanisms that led to obtain reduced values in Ca/P ratios when the oestrogenic deficiency was linked to alcohol consumption are not as yet well understood. However, both conditions have been separately associated with increased expression of important osteoclastogenics cytokines as IL-1, IL-6 and TNF-α.[28–31] Additionally, it is possible that there is also interference in the regulation of the RANK/RANKL/OPG, which may occur through increased expression of RANKL and decreased expression of OPG, leading to changes in the bone remodelling process with increased bone resorption.[31–33] It is also important to consider the possible toxic effects of excessive alcohol consumption on osteoblastic activity, a factor that could impair the process of bone formation and mineralization.[34,35]

The degree of mineralization can be modified by changes in osteoblast and osteoclast activity during the remodelling process. In cases concerning the changes in the rate of remodelling, with a predominance of the reabsorption process, as occurs in osteoporosis, there is not enough time for osteoblasts to complete the process of mineralization before the bone is reabsorbed prematurely by osteoclasts. These factors could affect the degree of bone mineralization and consequently the Ca/P ratios.[6,7,36]

In the present study, only the association of alcohol with oestrogenic deficiency was able to significantly decrease the Ca/P ratio in alveolar bone. The alcohol alone was not capable of promoting such changes. Similarly, Trevisiol et al.[37] found that alcohol consumption (ethanol contributing to 35% of caloric intake) did not impair mineralization in a model of osteoinduction in rats.

Theoretically, it was expected that ovariectomized rats could present a tendency to decrease the percentages of minerals, such as Ca and P, due to increased bone remodelling process, with a predominance of resorption and decreased bone mineral density.[6,7,31,36,38] In the present study, ovariectomized rats receiving a controlled diet (Ovx/alc and Ovx/iso) presented decreased percentages of Ca and P. However, it did not occur with rats Ovx/ad libitum, the group where the highest values of Ca/P ratios were found.

In the present paper, the Ovx/ad libitum group gained more weight and consumed more food. Other authors also observed an increase in body weight in ovariectomized rats when compared to sham operated groups.[39–41] Ovariectomy may increase food intake and weight gain, and studies show that treatment with estradiol reverses these effects.[39,42] Studies with knockout animals (for oestrogen receptor-alpha and aromatase) found that oestrogen may be important for the maintenance of lipid homeostasis.[43,44]

The results of this experiment do not allow for elucidating the reasons leading to higher values in the Ca/P ratios in the alveolar bone crest of ovariectomized rats who received ad libitum diet, compared to other groups exposed to oestrogen deficiency. However, one possible explanation may rely on the fact that the Ovx/ad libitum rats consumed significantly more food than those with dietary control. There is evidence that exercise may be related to increased bone mineral density in postmenopausal women[45] and likewise, with increased food intake, there was a higher incidence of bite forces on the alveolar bone, which may have led to a change in bone mineral density locally, as suggested earlier.[38,46] Patullo et al.,[46] suggested that the incidence of normal occlusal forces could promote protection against the development of osteopenia in the mandible.

Additionally, the increased food intake by the Ovx/ad libitum group also resulted in a higher consumption of key nutrients to maintain bone quality, including Ca and P.[47] This fact may also help to explain the high values in the Ca/P ratios found in this group. Another explanation may be the influence of weight gain on bone tissue. Some researchers suggest that, after menopause, heavier women conserve more bone mass when compared to women with lower body weights.[48,49] Leptin, a cytokine secreted by fat cells, has been studied as a potential modulator of the protective effects of fat mass on bones.[49]

A possible influence of increased food intake, higher incidence of bite forces and weight gain resulting in the highest values of Ca/P ratios in the group Ovx/ad libitum can be considered a hypothesis as an explanation to the result which was not theoretically expected. However, without further analysis, an equally possible hypothesis is that in the absence of other factors, oestrogen deficiency actually correlates with increased alveolar bone mineralization. It is important to consider that other numerous factors could also influence the progression of periodontal disease and possibly the quality of alveolar bone and tooth retention. Some of these factors include bacterial biofilm, systemic diseases, genetic disorders, habits, age, gender, stress and nutritional problems.[11–18]

It is also important to note that, despite the Ovx/ad libitum group showing the highest average in Ca/P ratios, which was statistically different from other ovariectomized groups (Ovx/alc and Ovx/iso), it was not different from Sham/ad libitum. Thus, from the results of this study, it was not possible to conclude that ovariectomy alone (without an associated dietary treatment) was able to significantly change the stoichiometry of hydroxyapatite on alveolar bone.

Some authors suggest that dietary changes might interfere with the host's response to periodontal disease progression.[17,50,51] Possibly, this is related to the fact that chronic inflammation causes oxidative stress, with an increased demand for a mineral and antioxidant vitamin rich diet.[17,50] In this study, it was found that the lowest values in Ca/P ratios were obtained in groups with dietary control (Ovx/alc, Ovx/iso, Sham/alc and Sham/iso), and were higher in groups with the ad libitum diet (Ovx/ad libitum/Sham/ad libitum). These findings suggest that diet may play an important role in the variations of the stoichiometric hydroxyapatite. However, further studies are necessary to validate this statement with greater statistical reliability.

Within the alcohol groups of the present study, an average of 37.83% of total calories came from alcohol, similar to previous studies, in which alcohol was responsible for 35–40%

of calories in the rats' diet.[28,52,53] This is considered a high dosage of alcohol consumption,[28,52,53] resulting in elevated blood ethanol concetrations.[52] In another study[35] with rats treated with 20% ethanol (in drinking water), similarly to that undertaken in our study, blood alcohol levels were eight times higher in the treated rats (0.869 g $l^{-1}$) than those in the control group (0.11 g $l^{-1}$). The 20% concentration was administered for 15 days which was considered a chronic intake.[35] In our study the rats received alcohol for eight weeks.

A comparison of results suggests that our rats were subjected to an excessive and chronic consumption of alcohol. This is an important factor, as most researchers seem to believe that the harmful effects of alcohol on bone is observed with abusive alcohol consumption and not with moderate consumption.[54,55]

The methodology for the treatment of the animals is based on previous studies[21–23] that have used similar experimental groups, concentration of alcohol (20% in drinking water) and time of ovariectomy. The standardization of the treatment facilitated the comparison of our results with other studies.[21–23] However, potential confounding effects pertaining to the type of diet used in our experiment should be considered when interpreting the results. Lieber et al.[56] criticized the delivery of alcohol in drinking water, as it reduces water intake and makes it difficult to control nutrients. Since nutritional changes could interfere with the host's response to the progression of periodontal disease,[17,50] other studies could verify if the results of this experiment would be similar if other forms of administration of alcohol could be considered, for example, using a nutritionally adequate liquid diet containing alcohol (Lieber–DeCarli liquid diet),[28,37,53,56] the administration of alcohol by intraperitonial injections[27,32] or by intubation.[57]

The present study has some limitations. One of the limitations was that of not being able to control the isocaloric group ingesting exactly the same amount of calories as those of the alcohol group (when considering the liquid diet). To minimize this problem, other ways of administrating the liquid diet could be considered. However, this limitation did not appear to have directly influenced the results, as no significant differences in Ca/P ratios were found between the isocaloric groups and control group (Sham/ad libitum). However, in relation to solid food, which provided the nutritional intake of Ca and P, nutritional pairing was achieved. Even though controlling the amount of alcohol consumed by the animals was achieved, another limitation of our experiment was the absence of evaluating blood alcohol concentrations. Other studies could include the measurement of this in their experimental designs. Finally, it is important to consider that our work was limited to Ca/P ratio analysis. Without other parameters of evaluation, it was only possible to correlate the results with other searches. Broader studies are therefore required to better verify the potential relevance of these results in dental practice.

## 5. Conclusion

It can be concluded that ovariectomy associated with alcohol consumption of 20% led to a significant decrease in Ca/P ratios within the region of alveolar bone crest in rats.

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